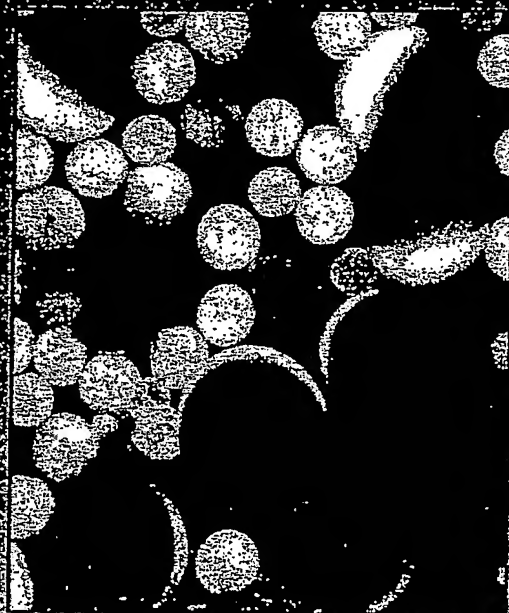


Methods in Molecular Biology

Volume 62

RECOMBINANT GENE EXPRESSION PROTOCOLS

Edited by
Rocky S. Tuan



 Humana Press

RECOMBINANT GENE EXPRESSION PROTOCOLS
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METHODS IN MOLECULAR BIOLOGY™

Recombinant Gene Expression Protocols

Edited by

Rocky S. Tuan


Thomas Jefferson University, Philadelphia, PA

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Strategies in Generating Transgenic Mammals

Olena Jacenko

1. Introduction

The ability to manipulate genes in mammals is providing insights into most aspects of modern biology, including the regulation and function of genes, the mechanisms of developmental and pathological processes, and the generation of animal models for human disorders. Furthermore, the development of gene transfer techniques is stimulating efforts to treat human diseases with gene-based therapies, and is establishing a new area for biotechnology in which transgenesis can be used for the improvement of domestic animals and plants, as well as for the production of rare products. The focus of this chapter will be to provide an overview of the strategies that can be used to alter the mammalian genome through gene transfer. Advantages and disadvantages of each approach will be discussed, and specific examples of how each strategy can be applied to address problems in mammalian biology will be provided in order to illustrate the potential scope of transgenesis.

A transgenic animal is defined here as one whose genome contains DNA of exogenous origin that has been introduced through experimental manipulation. By this definition, all animals with an *experimentally* altered genome resulting either from microinjection of recombinant DNA, infection with recombinant retroviruses, replacement of pre-existing genes with inactivated or mutated variants by gene targeting, or introduction of altered multipotent stem cells (e.g., hematopoietic, liver), are transgenic. Likewise, a transgene is the exogenous DNA introduced through experimental manipulation into the animal's genome, and includes recombinant DNA or retroviral constructs used for microinjection/infection, as well as replacement and insertional vectors used for gene targeting.

2. Establishment of Methods for Manipulating Genes in Mammals

Historically, the mouse has been the mammal of choice for genetic analysis because of its size, short gestation period, relatively large litters, availability of inbred strains, and its numerous spontaneous mutations mimicking human genetic diseases. Furthermore, mouse embryology and immunology have been extensively studied. For these reasons, it is not surprising that the development of transgenic and embryonic stem cell technology was first achieved in the mouse, rather than in less complex organisms such as flies, worms, or fish. The first transgenic mouse was generated by Jaenisch and Mintz in 1974 (1), when the simian virus 40 (SV40) DNA was microinjected into the blastocyst cavity of mouse embryos. Subsequently, germ line transmission of retroviral DNA was demonstrated following the exposure of early mouse embryos to a solution containing infectious retroviruses (2,3). Infection of mouse embryos with recombinant retroviruses however, constitutes only one of at least six methods of transgene transfer (Fig. 1). The most commonly used technique to date involves direct microinjection of recombinant DNA into the pronucleus of a fertilized egg (4). Although considerable work occurred in the 1960s and 1970s to provide a foundation for the development of transgenic mammals (*see ref. 5 for review*), the first report describing the presence of microinjected sequences in newborn mice appeared in 1980 (6). The detailed protocol for gene transfer through microinjection remains virtually unchanged to date (4), and represents the method through which the majority of transgenic mice are produced. Immediately following this initial report, five laboratories demonstrated stable integration of microinjected DNA into the host chromosome, and the expression of these genes in embryos and mice (7–11). The accelerated growth of mice carrying a metallothionein-growth hormone fusion gene provided the most dramatic demonstration that the integrated genes were expressed and functional (12). The genetically-engineered mice were termed “transgenic” (*see ref. 5, 13–18 for background*).

The almost parallel development of a complementary approach involving gene targeting, the process of homologous recombination between an introduced altered gene and an endogenous chromosomal allele, has greatly facilitated the studies of mammalian development. The transfer of DNA into totipotent embryonic stem (ES) cells, which are capable of contributing to the germ line when reintroduced into the host, has been widely used to overexpress or inactivate genes both in cell culture and in vivo (19–24). The basis for this technique stemmed from experiments performed as early as the 1960s with teratocarcinoma cells (25), embryonal carcinoma cells (26), as well as hematopoietic multipotent stem cells (5,16,17), and was greatly enhanced by the development of transgenic techniques during the 1980s. The current strategy for germ line modification through homologous recombination, as well as a

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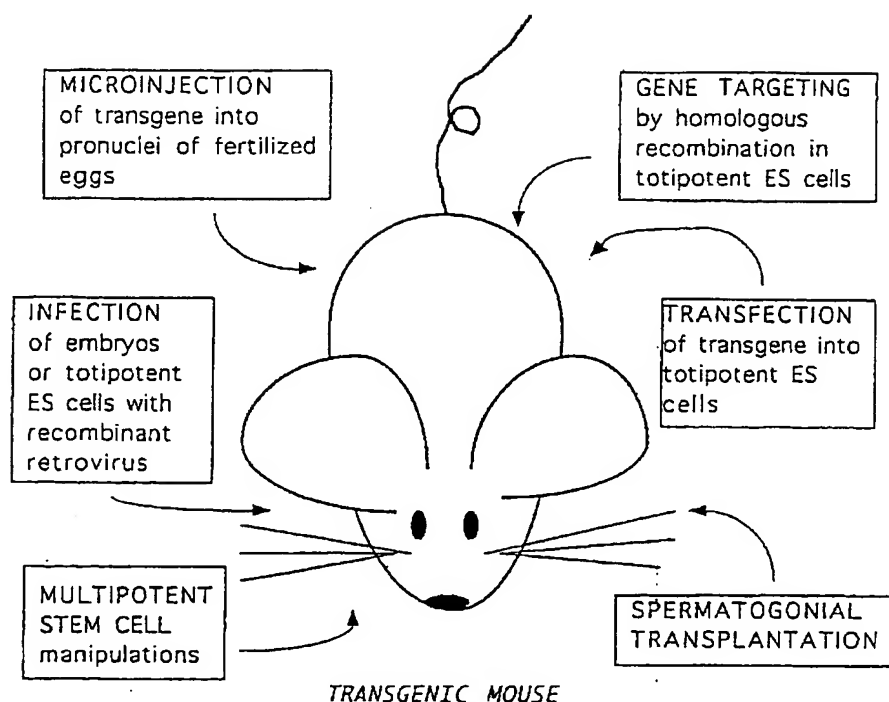


Fig. 1. Strategies for introducing transgenes into mice.

method for enrichment of ES cells in which the desired targeting event has occurred, are outlined by Capecchi and coworkers (20,27). Thus, within one decade, significant advances were made in the ability to study gene regulation and function in the context of a whole animal; the technology became available for producing an animal with a variety of desired genotypes by experimental means. Adding to this progress, Brinster and coworkers recently described a novel method involving spermatogonial transplantation (28–30), which offers the potential for transgenesis. The current strategies for generating transgenic mammals:

1. Microinjection of transgene into fertilized eggs;
2. Infection with recombinant retroviruses;
3. Gene targeting in ES cells;
4. Manipulation of multipotent stem cells; and
5. Spermatogonia transplantations include the following and are summarized (Fig. 1).

3. Transgenesis by Pronuclear Microinjection of Recombinant DNA

3.1. Methodology

The technique described by Gordon et al. (4) remains the method of choice for dissecting the intricate regulatory elements governing gene regulation, and

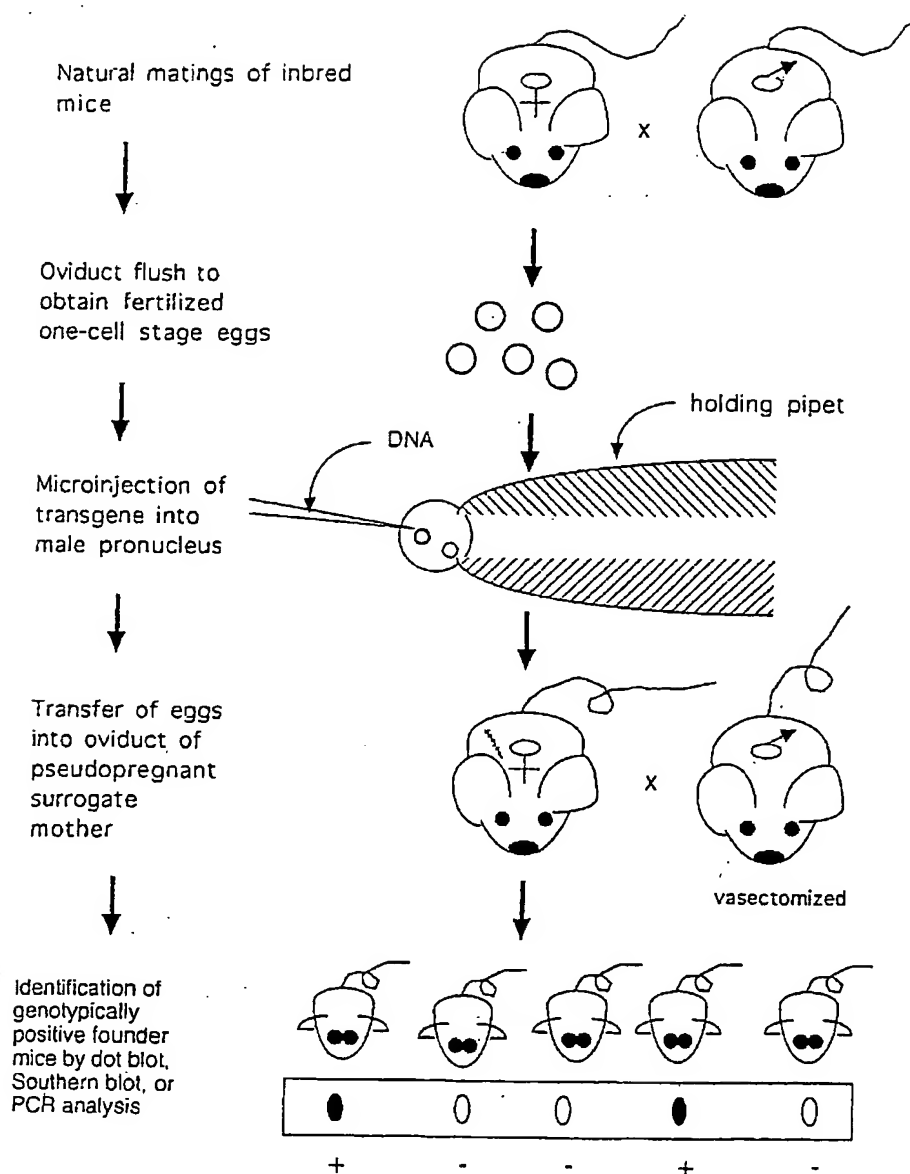


Fig. 2. Generation of transgenic mice through pronuclear microinjection of transgene. See text for details.

for expressing a given gene in almost any tissue (Fig. 2). Briefly, freshly isolated fertilized mouse eggs at the one-cell stage are cultured for 1–2 h until the pronuclei become visible. A solution containing the transgene of interest (present in linearized form with minimal vector sequences) is then microinjected via a glass micropipet into the male pronucleus of a fertilized egg that is restrained; a successful injection is evidenced by pronuclear swelling. The injected eggs are then surgically transferred into the oviduct of a pseudopregnant surrogate mother, who has been previously mated with a vasectomized

male. The resultant pups (f_0) are analyzed for the presence of the transgene by either genomic Southern blotting, dot blotting, or PCR, using DNA obtained from tail biopsies. Typically, approx 10–20% of the pups born carry the transgene (14,31,32). Each animal positive for the transgene is referred to as a founder, and represents the result of an independent transgene microinjection and integration event. The founders are bred with wild-type mice to obtain offspring (f_1) that also carry the transgene, thereby establishing unique families of mice, or transgenic lines. If germ line transmission is achieved, the interbreeding of f_1 hemizygotes (carrying the transgene on one of the two chromosomal alleles) gives rise to a portion of mice homozygous for the transgene. Homozygotes are identified by the intensity of transgene hybridization signals on genomic Southern blots; furthermore, they are “proven” homozygotic by backcrossing with wild-type mice, which should generate 100% hemizygous offspring. Genotyping and expression analysis of these mice are essential for determining if and where the transgene is expressed, and whether the transgene segregates with the observed phenotype. Furthermore, the integration site of the transgene in the chromosome also may influence the pattern and level of expression, necessitating the analysis of at least two transgenic lines per transgene construct. Although transgene expression is often stable over a number of generations, rearrangements and deletions may occur (7,33), and should therefore be screened for. These critical issues are discussed in Section 3.3.4.

Transgene integration will usually occur at the one-cell stage; therefore the germ cells and all somatic cells of the founder will contain the foreign DNA. However, if integration occurs at a later point, not all cells may have the transgene. In such a case, the mouse is a mosaic for the transgene (31). Furthermore, transgene integration is random; therefore the DNA may insert anywhere in the genome, and by doing so, may disrupt endogenous gene function, leading to a phenotype. Approximately 10% of the random integration events result in insertional mutagenesis, which most commonly manifests as a recessive phenotype (4,34). This event represents one unexpected and major benefit of gene transfer through microinjection, as well as through retroviral infection; transgenes can act as insertional mutagens that can inactivate and thereby identify endogenous genes involved in specific developmental processes (see Section 3.3.5.) (34,35).

It is also possible to microinject a transgene construct that will express a protein that will contribute to a phenotype independent of the integration site (Fig. 3). To result in transgene expression, the transgene construct must include a promoter. This promoter may be constitutive, inducible, cell-specific, viral, or that of a housekeeping gene. Likewise, the transgene whose expression is driven by this promoter may either be a reporter gene whose activity can be

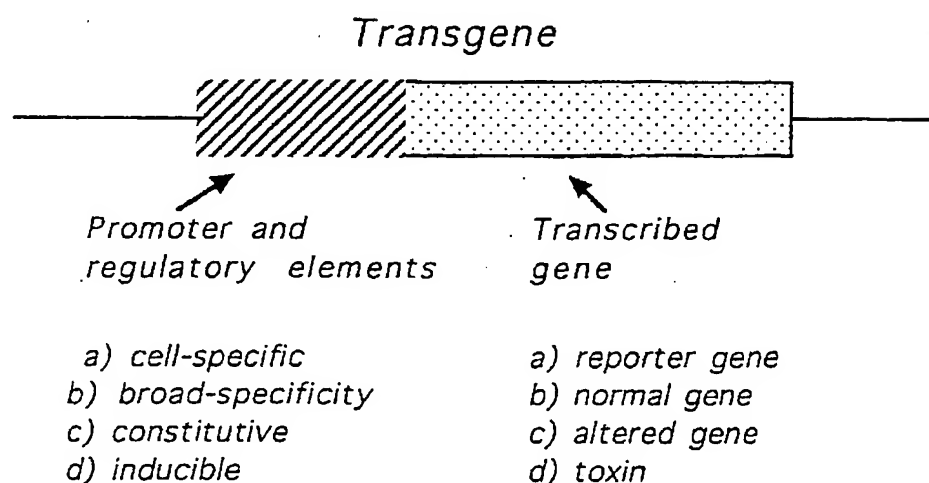


Fig. 3. Transgene construct for pronuclear microinjection into fertilized eggs. A hybrid construct can be designed by combining different types of promoters, which will drive the expression of a variety of genes to address gene regulation or function.

monitored histochemically or enzymatically (such as β -galactosidase, luciferase, chloramphenicol transferase), a normal or an altered mouse gene, a gene from a different species, or even a synthetic gene. This type of a "hybrid" transgene construct can be designed to address issues concerning either gene regulation or function.

3.2. Gene Regulation Studies

Many early studies using transgenic mice generated by pronuclear microinjections were designed to address the control and tissue-specificity of gene regulation (36–40). By altering the nature and extent of the transgene promoter and by monitoring the expression of a reporter gene, regions within the promoter that are required for temporal and cell-specific expression could be mapped. These reporter genes are often, but not always, of prokaryotic origin and usually encode proteins that are not typically expressed in most eukaryotic cells. The expression pattern of the selected reporter gene, and thus the specificity of the promoter, can be determined either histochemically, by *in situ* hybridization, or biochemically in tissue homogenates (40). For example, among the most commonly-used reporter genes is *lacZ*, encoding for bacterial β -galactosidase. *LacZ* activity can be successfully localized in mouse embryos by incubating whole embryos with X-gal, a substrate for β -galactosidase that is converted to a deep blue product (38–41). However, *lacZ* staining in post-natal mice often yields unreliable results owing to nonspecific staining, in which case alternate reporter constructs may be designed. The activity of reporter genes such as firefly luciferase or bacterial chloramphenicol trans-

ferase (CAT) could be detected in tissue extracts in the presence of the appropriate substrate either spectroscopically (for luciferase), or biochemically (for CAT) (38,40).

It is important to note that results obtained from such *in vivo* analyses in transgenic mice do not always mimic those obtained from *in vitro* assays. Since most data on gene regulation is generated through transient transfection expression analyses, it suffers from the general shortcoming of such an approach, namely, the absence of proper chromatin structure for controlled expression of the transfected DNA. Furthermore, many transfection studies are being carried out in cells that have no endogenous expression of the gene of interest (owing to the difficulty in isolating and culturing certain cell types), or in cells from different species, making the data difficult to interpret (42). The availability of an *in vivo* approach has been essential for testing the *in vitro* observations, and has confirmed the identification of transcription regulatory regions as major determinants of tissue-specific gene expression in the whole organism. To date, transgenesis by pronuclear microinjection of promoter-reporter constructs remains the most successful strategy for mapping regulatory elements in genes. The importance of identifying regulatory elements and knowing how genes are controlled is a prerequisite for targeting genes to specific sites, and is of utmost relevance for gene therapy.

3.3. Strategies to Study Gene Function

The ability to express genes in selected cells and tissues has led to even more profound possibilities: transgene products could interfere with specific gene functions or protein interactions in complex systems, and consequences of these alterations could be monitored (15,16,43,44). Through design of a hybrid transgene construct comprised of a tissue-specific promoter linked to a normal or altered gene of interest, the deregulated transgene expression may yield insights into gene and/or tissue function. Several strategies outlined can be used for analysis of gene function using transgenic mice:

3.3.1. Antisense RNA

This strategy involves blocking the expression of an endogenous gene by preventing translation of sense transcripts. In principle, the antisense approach is possible, and has had some success *in vitro* (45,46), as well as in the generation of transgenic flies (47) and frogs (48). In practice, its use has been limited in mammals, although some success has been documented (49,50).

3.3.2. Dominant Interference; Dominant Negative

A more powerful approach involves generating a dominant interference phenotype in transgenic mice, by blocking the function of a gene at the protein

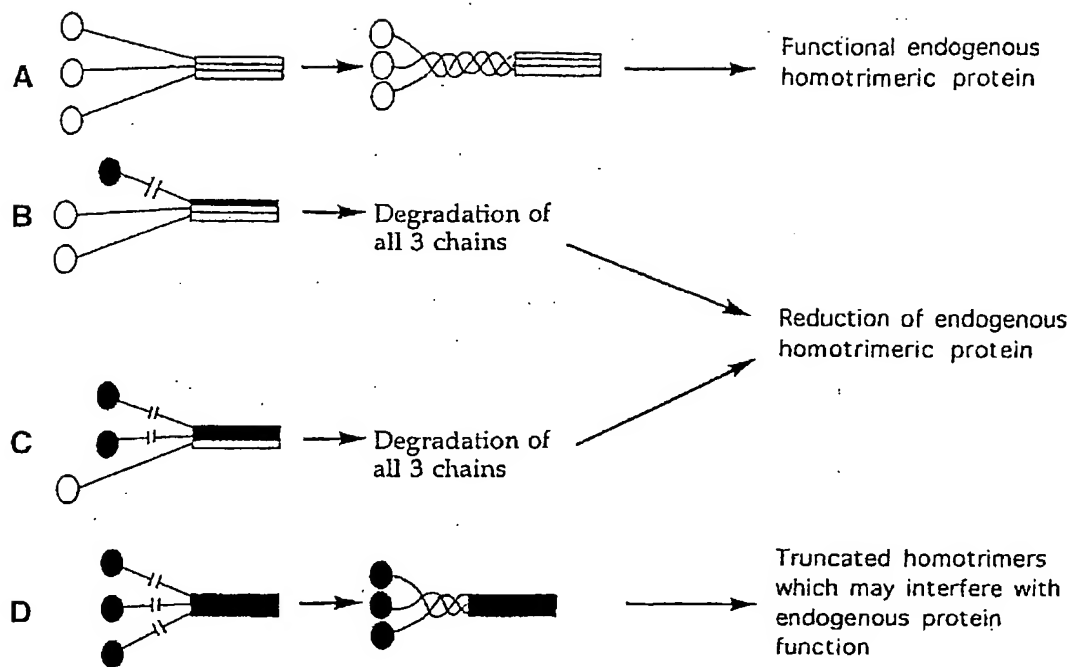


Fig. 4. Schematic representation of dominant interference. Expression of a transgene encoding truncated polypeptides (shaded molecules) results in a competition with the endogenous polypeptides (clear molecules) for binding, followed by the inability of hybrid molecules to form stable trimers (**B** and **C**) unlike endogenous molecules (**A**). Such a scenario will likely lead to degradation of the hybrid chains through a protein suicide mechanism, leading to a partial or complete loss of function of the endogenous gene. Truncated homotrimers may also persist and interfere with the function of wild-type trimers (**D**). This may contribute to a loss of function phenotype, but may also result in gain of function. See text for discussion. (This diagram is adapted from ref. 70.)

level through expression of an inhibitory variant of the same protein (43). This approach is particularly effective for multimeric proteins (e.g., collagens), proteins with multiple functional domains or subunits (e.g., gene-regulatory proteins), or enzymes whose activity is limited by substrate availability (Fig. 4). The resultant phenotype is considered dominant, because even very low levels of the inhibitor will have an effect (usually disruptive) on the normal function of the endogenous protein. A dominant negative phenotype may result from a partial or complete loss of function of the endogenous gene product. An excellent example of this approach is provided by work on collagens (see references within refs. 51,52), where the first dominant negative mutation was introduced within the *Colla1* gene (53). Type I collagen, the most abundant structural extracellular matrix protein predominantly found in dense connective tissues, has been associated with the inherited disease osteogenesis imperfecta in

humans (54). Transgenic mice bearing single residue substitutions within one type I collagen gene developed a dominant phenotype characteristic of the human disease, and demonstrated that as little as 10% of mutant gene expression was needed to disrupt folding of collagen chains into functional trimers (53).

It is important to realize, however, that although the dominant interference approach is designed to disrupt endogenous gene function at the protein level, overproduction of an inactive or a modified protein can have an opposite effect leading to a new phenotype or a gain of function. It is also noteworthy that many naturally occurring mutations may function by dominant interference, resulting in loss and/or gain of function phenotypes.

3.3.3. Overexpression

The second successful approach involves overexpressing a transgene product in appropriate or inappropriate cells to create an imbalance in the concentration of the correct gene product. Often, this will create a competition situation (as in dominant interference) between the transgene product and the endogenous protein, leading to a dominant negative, and a loss of function phenotype. However, there are also examples where this approach has led to a new phenotype as a result of a gain of function. One example of this approach is provided by the deregulated expression of the proto-oncogene *c-fos* by Wagner and coworkers (32). Expression of *c-fos* in a number of transgenic mouse tissues has resulted in an effect only in bone and the thymic epithelium, identifying the cells within these tissues as the targets for *c-fos* action. Such results from *c-fos* overexpression studies are currently enabling the investigators to unravel the complex pathways leading to oncogenic transformation. An additional dramatic example involves the use of a broad specificity inducible promoter to express the human growth hormone gene in mice (12). The resultant "big mice" demonstrated the role of the growth factor in organismal growth, which has been subsequently used to correct growth deficiency in dwarfed mice (55).

3.3.4. Ablation of Cells

The strategy involving the selective destruction of cell types and tissues is summarized in the review by Hanahan (15). Briefly, the transgene construct is designed to consist of a cell or tissue-specific promoter linked to a toxin gene such as diphtheria toxin A or ricin (56–58). Such an approach has potential for addressing questions concerning cell function, lineage, and interactions during development (59). However, the use of toxins in transgenic mice has revealed a problem with penetrance of the transgene, resulting in limited cell death (56,57). A variation of this approach involved the design of a suicide vector containing a "drug-conditional" promoter, whose expression resulted in cell

death only when a drug was administered (60). However, one shortcoming of this method is realized when a herpes simplex virus thymidine kinase (HSV-TK) promoter is used; in such a case, cell death occurs only in proliferating cells through the incorporation of the drug gancyclovir, a nucleoside analog, into replicating DNA. Perhaps the recent cloning and characterization of apoptosis genes (61,62) may provide the necessary tools for cell- or tissue-specific ablation without the aforementioned limitations.

3.3.5. Insertional Mutagenesis

Insertional mutations arising from the random integration of defined DNA sequences are especially valuable for identifying genes with developmental roles. In this approach, the integrated DNA serves two purposes. First, the transgene disrupts the endogenous gene, leading to a mutant phenotype; second, it acts as a molecular "tag" marking the integration locus. By recloning the integrated sequences, the disrupted endogenous sequences can be recovered. Through random insertional mutagenesis, a number of genes with developmental effects have been cloned (34,63). One of the first examples involves the limb deformity mutation characterized by Leder and coworkers (64). While investigating the role of *c-myc*, several transgenic lines were generated and bred to homozygosity with respect to the inserted gene. In one of these lines, a recessive mutation has resulted in severe dysmorphism in limbs, and has thus provided a link with the control of pattern formation in the developing mammalian embryo. The first mutation arising from retroviral insertional mutagenesis involved the *Mov-13* transgenic line generated by Jaenisch and coworkers (65,66), which likely represents the most thoroughly characterized transgenic mice to date. In these mice, retroviral infection of postimplantation embryos resulted in a single proviral insertion into the first intron of the $\alpha 1(I)$ collagen gene, causing a recessive perinatal lethal phenotype. These mice are continuing to provide data on the role(s) of fibrillar collagens during embryogenesis and postnatal life, on collagen gene regulation, on fibroblast, osteoblast, and odontoblast cell lineages, and on angiogenesis.

A prerequisite to the insertional mutagenesis approach is the demonstration that the mutant phenotype results from transgene disruption of an endogenous gene (see Section 3.3.6.), as well as the isolation of the altered gene. One complicating factor in cloning the disrupted gene when insertional mutagenesis results from the pronuclear injection of the transgene, stems from transgene integration in tandemly repeated copies, as well as potential rearrangements of the endogenous gene near the integration sites (7,14,33). To facilitate the screening and isolation of genes involved in morphogenesis, new strategies involving "enhancer traps" and "gene traps" were developed for "tagging" the mutated genes of interest (63,67-69). The transgene constructs used in these

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strategies both carry a gene for β -galactosidase, and are electroporated into pluripotent ES cells, which are subsequently reintroduced into the blastocyst (see Section 5.). As mentioned previously, *lacZ* expression is observed histochemically by staining early embryos with X-gal, a substrate for β -galactosidase that is converted to a deep blue product. In "enhancer traps," *lacZ* is linked to a weak promoter, and expression is dependent on the vector's integration near an enhancer. In "gene traps," the *lacZ* gene lacks regulatory sequences except for a splice acceptor site; expression is only achieved if the vector integrates into an intron of a cellular gene, and if splicing results in a chimeric mRNA that produces a functional fusion protein. Thus in both cases, the *lacZ* transgene constructs are used to rapidly screen many integration events, and to identify and clone regions of the mouse genome that are active in a temporal and spatial pattern during development (67).

3.3.6. Analysis of Transgenic Mice

The basic requirement in the analysis of transgenic mice is to establish the involvement of the transgene in any newly identified alteration in phenotype. First, to exclude a spontaneous mutation arising coincidentally in the transgenic strain as the cause of the phenotype, the observed murine phenotype and the transgene must be demonstrated as genetically inseparable. This is accomplished by standard genetic crosses and genomic DNA analysis by Southern blotting and/or PCR to identify genotypically positive pups, and to monitor transgene cosegregation with the observed phenotype. Second, to establish whether the phenotype results from insertional mutagenesis or from transgene expression, mice from several transgenic lines (carrying the same transgene) need to be compared based on genotype, phenotype, and transgene expression. To rule out insertional mutagenesis, a minimum of two lines (representing at least two independent microinjection events and transgene insertion sites) must show the same phenotype and express the transgene message/product in a similar temporal pattern. Southern blot analysis of genomic DNA obtained from tail biopsies from mice in these lines should reveal different insertion sites, evidenced by differences in migrations of specific DNA fragments following digestion with the same restriction enzyme (e.g., ref. 70; Fig. 2A). Southern analysis will also reveal transgene dosage, as well as head-to-tail arrangements. It is important to realize that transgene expression does not necessarily correspond to transgene copy number, but is influenced by the insertion site microenvironment. Furthermore, transgene deletions and rearrangements can occur over a number of generations, and thus may influence transgene expression and the resultant phenotype. These points further underline the importance of initially analyzing mice from several transgenic lines, and then maintaining two-to-three of these lines for characterization.

If more than one transgenic line is not available, or if the phenotype appears to result from insertional mutagenesis, characterization of the transgene insertion site becomes necessary. This is accomplished by first cloning the genomic DNA flanking both sides of the inserted transgene, and later using these clones to isolate the intact gene. As mentioned previously, a significant drawback of gene transfer through pronuclear microinjection stems from transgene integration in multiple head-to-tail concatenated arrangements; these long blocks of tandemly repeated copies often are larger than the capacity of standard cloning vectors, making it difficult to clone the junction sites. Furthermore, complex rearrangements are also known to occur at the integration point, complicating gene isolation. To circumvent this potential problem, a cosmid library and cloning vector may need to be generated. Subsequent mapping of the integration site to a chromosome would permit comparison of this locus to that of other known genes, or mapped mutations. Identification of similar mutant phenotypes near this locus may lead to genetic complementation tests (64), and may provide conclusive proof for insertional mutagenesis.

3.3.7. Summary

3.3.7.1. ADVANTAGES OF GENERATING TRANSGENIC MICE THROUGH PRONUCLEAR MICROINJECTION

1. This represents the most successful approach to date for mapping regulatory elements in genes.
2. The design of hybrid promoter-transgene constructs permits expression of exogenous DNA in virtually any site.
3. The dominant interference strategy may provide insights into specific protein action and mechanisms of pathogenesis, and requires only the expression of a mutant gene product rather than the inactivation of an endogenous gene. Furthermore, such mutations may be representative of many heritable disorders.
4. Random insertional mutagenesis may lead to the identification of developmentally active genes.
5. There is no restriction with respect to size or type of DNA, which is microinjected (unlike the case with retroviral vectors and gene targeting through homologous recombination).
6. This represents the only means to date of generating transgenic domestic animals (18).

3.3.7.2. DISADVANTAGES

1. The random introduction of exogenous DNA into the genome may result in an unexpected (and often complex and difficult to interpret) phenotype, independent of the desired effect.
2. Gene integration is not targeted; therefore, it is difficult to predict tissue-specific expression; this is a significant concern for gene therapy.

3. Data relating to gene function may be difficult to interpret; although the transgene construct may be designed to disrupt endogenous gene function, the overproduction of an inactive product may have the opposite effect.
4. The integration site is difficult to clone owing to transgene tandem arrays and gene rearrangements (this is circumvented by using retroviral vectors).
5. Pronuclear microinjections require the availability of expensive microinjection facilities, as well as technical experience unavailable to most laboratories. Thus, government subsidized programs (such as DNX, Princeton, NJ) and university core facilities are beginning to extend these services. Nevertheless, the establishment, characterization, and maintenance of transgenic lines in virus-free animal facilities remains a labor-intensive and expensive process.

4. Transgenesis by Retroviral Infection

4.1. Methodology

One advantage of using viruses (most frequently recombinant retroviral vectors) for transgene introduction either directly into embryos, into ES cells that can then be used to form chimeras, or into multipotent stem cells that can replace an endogenous tissue, is the technical simplicity of the protocol. Briefly, stem cells or embryos at various stages of development are infected at an efficiency approaching 100% by their coculture with cells producing the virus. The efficient infection and expression in a wide variety of cells represent the fundamental advantage of using retroviruses. Furthermore, stable and accurate integration of a single viral transgene copy into the host DNA facilitates the identification of the insertion site. For this reason, viruses are superior agents when genetic tagging of chromosomal loci by insertional mutagenesis or when marking cell lineages in stem cell differentiation and during embryo development is desired (35,71). The disadvantages include size constraint of the retroviral vectors, with the insert size being no larger than ~8 kb of DNA (and in some cases significantly smaller), precluding the expression of many genomic DNAs. Furthermore, infection by retroviruses requires cell replication; therefore, nondividing cells cannot be targets. Relatively low virus titers and low expression of the inserted genes are also problematic, as well as the instability of the retroviral vector structure. Finally, proper functioning of regulatory elements of the inserted DNA may be affected when positioned close to the viral long terminal repeats, altering cell-specific expression. Nevertheless, retroviral infection remains the only means at present through which DNA can be stably introduced into many somatic tissues and multipotent stem cells (*see* Section 6.) (14,16,18,35,71-74), and thus holds great promise as a strategy for gene therapy.

4.2. Summary

4.2.1. Advantages of Generating Transgenic Mice Through Retroviral Infection

1. Simplicity of protocol; no need for embryo microinjection or extensive ES cell screening in culture.
2. Most proliferating cells can be infected.
3. Integration of a single transgene copy occurs in transcriptionally active regions of the genome, and does not include chromosomal rearrangements. This facilitates the identification of the insertion site, and is ideal for tagging chromosomal loci and marking cell lineages.
4. Retroviral infection represents the only means of introducing a transgene into certain somatic cells.

4.2.2. Disadvantages

1. Exogenous DNA is randomly introduced into the genome and may complicate data interpretation.
2. Retroviral vectors have an insert size constraint.
3. Expression of the transgene is often poor.
4. Retroviral vectors are often unstable.
5. The juxtaposition of viral regulatory elements with those of the insert is problematic.

5. Transgenesis by Gene Targeting Through Homologous Recombination in ES Cells

Gene modification through targeting allows the derivation of mice with a predesigned genetic composition. This strategy has a major advantage over other transgenic approaches for the analysis of gene function, because rather than introducing exogenous DNA into the genome, the endogenous gene is replaced with a modified cloned gene during homologous recombination (HR) in cultured ES cells. In principle, any gene can be modified in a defined manner in any species from which ES cells can be obtained, and upon their reintroduction into the animal, the specific effects of the introduced modification can be observed (20,24,75).

The finding that ES cells can link genetic manipulations in vitro to analysis of function in vivo was pivotal in the establishment of gene targeting through HR (5,20). The ES cells are derived from the mouse blastocysts (3 d postcoitus), specifically from the inner cell mass from which the embryo develops. These pluripotent cells can be explanted and maintained as stable diploid cell lines under well-defined conditions (76). A targeting vector containing the desired gene mutation and two selectable markers, the bacterial neomycin-resistance gene (neo) and the Herpes Simplex virus (HSV) thymidine kinase (TK) gene (Fig. 5), can be introduced into ES cells by electroporation or microinjection.

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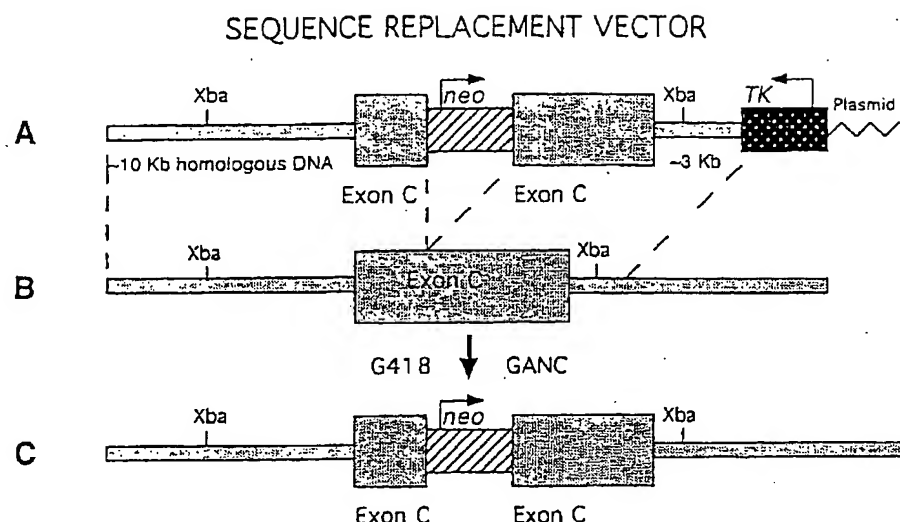


Fig. 5. Homologous recombination through use of a replacement vector. (A) Sequence replacement vector containing ~10 kb of homology with the endogenous locus, and ~3 kb 3' of the *neo* insertion, which interrupts the coding sequence within exon C. The genomic sequence is flanked on the 3' end by *TK* sequences. Arrows indicate transcriptional orientations of the *neo* and *TK* promoters; dotted lines indicate the regions of homology within which recombination may occur. (B) The endogenous wild-type locus in the region homologous to the replacement vector sequences. (C) The predicted structure of the altered endogenous allele following homologous recombination with the replacement vector shown in (A). Through this process, the endogenous sequences are replaced by the vector sequences containing *neo*.

In most cells, this vector will insert randomly into the ES genome; in a few cells however, the introduced DNA will pair with the cognate chromosomal DNA sequence and transfer the mutation into the genome through HR. The frequency of this double crossover event is very low (at best one out of $\sim 3 \times 10^4$ ES cells electroporated), and appears to depend on the extent of homology between the exogenous and chromosomal sequences in the cells (for DNA with 2–4 kb of homology, the frequency is one out of $\sim 5 \times 10^7$ – 5×10^6 ES cells electroporated) (19,20,27).

Sequence replacement or insertion targeting vectors can be designed. For gene inactivation, a replacement vector has been more commonly used (Fig. 5). This vector should ideally contain approx 10 kb of DNA homologous to the target gene to increase targeting frequency. A *neo* gene is inserted, along with its promoter, into an exon of the target gene sequence, thereby serving as a mutagen as well as a selectable marker. A *TK* gene is also cloned into the vector adjacent to the 5' or 3' region of homology. The vector is linearized outside the region of homology, and HR results in the replacement of the

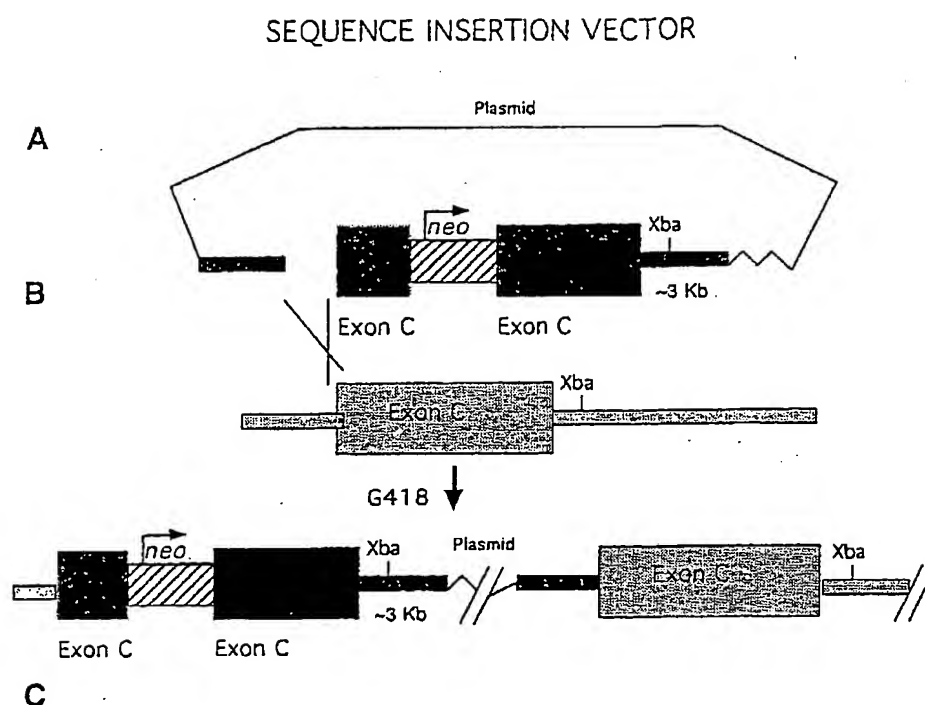


Fig. 6. Homologous recombination through use of an insertional targeting vector. (A) The sequence insertion vector, containing the recombinant DNA homologous to the endogenous locus (dark gray), with a *neo* insertion interrupting the coding sequence within exon C. Prior to electroporation, the vector is linearized within the region of homology, and the 5' and 3' ends lie adjacent to one another. (B) The endogenous wild-type locus in the region homologous to the insertion vector sequences. (C) The predicted structure of the altered endogenous allele following homologous recombination with the insertion vector shown in A. Upon pairing of homologous sequences and recombination, the entire vector is inserted into the endogenous gene. This procedure produces a duplication of the endogenous gene represented in the vector.

endogenous gene with the neo-containing genomic sequence; transfer of TK does not occur, because it lies distal to the region of homology. The insertion vector (Fig. 6) has been used both for gene inactivation and introduction of subtle site-specific mutations into the gene of interest (77,78). Prior to electroporation, the vector is linearized within the region of homology; HR results in the entire vector being incorporated into the endogenous gene, producing a partial duplication of the target sequence. Similar gene targeting frequencies have been reported for both types of vectors (27).

The rare ES cells carrying the targeted mutation are enriched by a positive/negative selection (PNS) procedure in culture (20) (Fig. 7). Briefly, ES cells are positively selected in G418-containing medium for clones with insertion of neo in their genome by either homologous or random integration. Negative

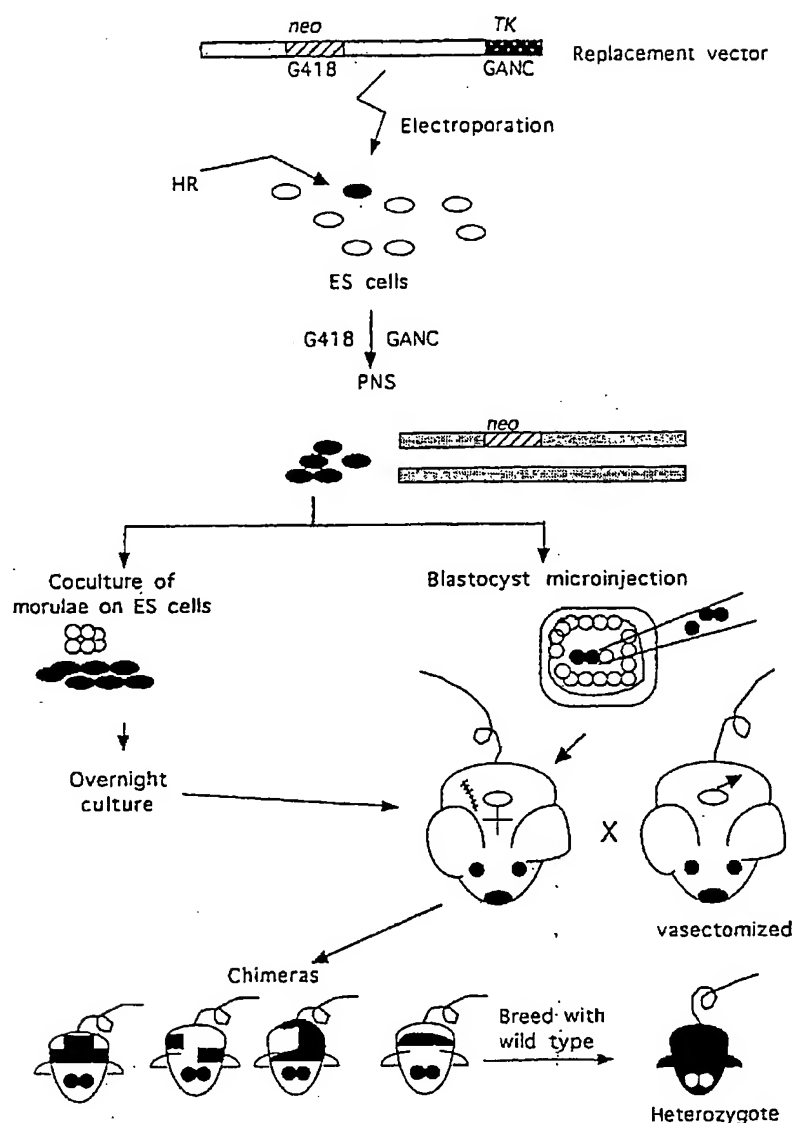


Fig. 7. Generation of germ line chimeras from embryo-derived totipotent stem cells carrying a targeted gene disruption. See text for details.

selection in the presence of gancyclovir (GANC) or FLAU selects against all clones with random integrations containing the TK product, thus enriching for cells containing the targeted mutation. The G418/GANC resistant clones are expanded, and screened by PCR and/or Southern blot analysis for the targeted DNA. The appropriate cells are then cloned and maintained as a pure population. Ideally, a minimum of two clones carrying independent HR events are selected for transfer into embryos. This is accomplished either by microinjection into blastocysts, or by mixing with morulas. Blastocyst microinjection (76), which has been the predominant method used to date, requires the avail-

ability of microinjection equipment, as well as experience with the technique. On the other hand, the recently described aggregation protocol (79) involves coculture of ES cells with morulae, which readily adhere. Using this technique, high success rates were reported (nearly 96% chimeric embryos) for ES cell incorporation in the inner cell mass, where they participate in somatic and germ cell development. The degree of embryo manipulation is also reduced, and the requirement for microinjection equipment is bypassed.

The ES cell-containing embryos are then surgically implanted into the uterus of a surrogate mother, where development proceeds to term. The resultant animal is a chimera, being composed of cells derived from both the donor ES cells and the host blastocyst. This is usually manifested by coat color, since chimeras have patches of both the color of the blastocyst strain, as well as the color of the host. Breeding of chimeras with wild-type mice tests for germ line transmission, and establishes heterozygotes (mice containing one allele with an altered gene, and the other with the wild-type gene). Interbreeding of such heterozygotes generates animals homozygous for the mutation (Fig. 7). Screening and analysis of animals is as described for transgenic mice; however, rather than testing for the presence and expression of the transgene, the absence of wild-type gene expression and function is assayed.

5.1. Transgenesis by Pronuclear Microinjection vs Gene Targeting by Homologous Recombination

Transgenesis (via pronuclear microinjection) and gene targeting are often directed toward different ends. As described previously, when studying function, the former can be used to introduce a foreign gene and to observe either its specific dominant effect on the endogenous gene, or the result of its deregulated expression. The latter strategy involving gene targeting can also be used to generate a gain-of-function scenario through ectopic overexpression; bypassing HR and PNS of the transgene (32). However, its most powerful use involves loss-of-function through inactivation of an endogenous gene. Among the many successful examples, the targeted disruptions of *int-1* (80,81) and *c-fos* (82) proto-oncogenes demonstrate the power of this technique. The *int-1* is temporally and spatially restricted during CNS development; its inactivation in mice resulted in the inability of portions of the brain to develop, implicating the role of *int-1* in the induction of the mesencephalon and cerebellum. The inactivation of *c-fos* has surprisingly resulted in osteopetrosis in mice. Through use of intricate hematopoietic cell culture and marrow transplantation assays, it was established that this proto-oncogene regulates the osteoclast-macrophage lineage determination, and thus affects bone remodeling (84,85). Among the many successes, however, there are also disappointments. The lack of an identifiable phenotype after gene inactivation has underlined the potential redun-

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dancy in gene function in nature. However, it is difficult to accept that certain genes are completely compensated for. It is more likely that we are unable to perform the proper diagnostic experiments (e.g., measure mouse intelligence, adaptability, and so on) to reveal the phenotype in all successful transgenic animal experiments.

5.2. Summary

5.2.1. Advantages of Gene Targeting Through HR

1. The genome is altered through the replacement of the endogenous gene with an altered one, rather than by random insertion of exogenous DNA; thus the precise consequences of mutations can be analyzed.
2. Although this method involves labor-intensive cell culture work, it is technically simpler than pronuclear microinjections.

5.2.2. Disadvantages

1. For increased targeting frequency, ~10 kb of homologous DNA are recommended, preferably from the same strain as the ES cells.
2. Gene inactivation occasionally results in either no detectable phenotype, or a very mild one, and is often considered not informative; caution should be taken in the interpretation of such data.

6. Transgenesis by Manipulation of Multipotent Stem Cells

6.1. Methodology

Strategies used for generating transgenic mice are not limited to experiments directed only at germ line modifications. In specific cases, it is advantageous to modify only certain somatic tissues of the organism via stem cells (e.g., hematopoietic, liver, epithelial, lung, etc.). As cell culture methods develop, it is becoming possible to use the available gene targeting protocols to correct defective genes in the appropriate tissues, leading to somatic gene therapy.

Two multipotent stem cell systems have been shown to be capable of repopulating their specific organs: the hematopoietic (16,17,62) and the liver (5,86) stem cells. Hematopoietic stem cells are multipotent in that they could regenerate cells of all the lymphoid and myeloid blood cell lineages, as well as produce more stem cells through self renewal. In vivo assays have been established where the regeneration capacity of these stem cells and their ability to maintain a functional hematopoietic system can be tested. These assays involve the removal of bone marrow (which represents the major site of blood production in the adult) from a donor animal, and its transplantation into a recipient host with a compromised or destroyed (by irradiation) hematopoietic system. The donor stem cells could also be modified through retroviral transgene transfer, enabling one to investigate specifically the effects of individual genes on

the well-characterized hematopoietic system, and to address the molecular mechanisms determining blood cell lineages (e.g., *c-fos*; 84). A detailed protocol for mouse hematopoietic stem cell infection by retroviruses is described by Wagner (16). It is also conceivable that introduction of altered hematopoietic stem cells into the blastocyst may modify selected cell lineages during development (5).

Likewise, the recent work of Brinster and coworkers on the regeneration potential of the liver promises tremendous opportunities. The unique Alb-uPA transgenic mouse model of spontaneous liver regeneration (86,87; reviewed in ref. 5) has demonstrated that transgene-expressing cells in the liver can be replaced early in life by endogenous cells that have deleted all functional transgene copies within the tandem arrays by chromosomal rearrangement. These studies indicate the remarkable regenerative capacity of the liver stem cell, as a clonal nodule can replace up to 95% of the normal liver mass.

In principle, the replacement of defective hematopoietic or liver cells may be achieved by foreign donor cells, by totipotent cells (whose totipotency can thus be tested in a restrictive environment), by multipotent cells from other organs, or by cells genetically engineered through retroviral infection to express specific genes. Furthermore, these approaches could be extended to stem cells in other systems. These techniques are summarized and discussed by Brinster (5). Such combinations of stem cell manipulation and transgenesis may greatly facilitate studies on organ development and cell lineage evolution, as well as on the assessment of the developmental potential of a variety of stem cells, and the establishment of therapies.

6.2. Summary

6.2.1. Advantages for Generating Transgenic Animals Through Manipulation of Multipotent Stem Cells

1. This strategy presents new possibilities for unraveling molecular mechanisms of cell differentiation.
2. The developmental potential of a variety of stem cells (multipotent as well as totipotent) may be assessed.
3. New applications for somatic cell therapies may be developed.

6.2.2. Disadvantages

These strategies target somatic cells, and will not influence the genotype of progeny.

7. Transgenesis by Spermatogonial Stem Cell Transplantation

Spermatogonia represent the only self renewing stem cell population in the body that is capable of germ line contributions. Recently, Brinster and

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coworkers have demonstrated the ability to harvest these cells from donor testes, maintain them in vitro, and microinject them into recipient testes. Such spermatogonial transplantation has resulted in normal spermatogenesis, and functional spermatozoa were produced that could fertilize eggs and give rise to offspring (28–30). These elegant studies establish a technique that may eventually lead to novel approaches for generating transgenic animals, and provides important implications for studies of embryo development, infertility treatment, and germ line gene modification.

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References

1. Jaenisch, R. and Mintz, B. (1974) Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. *Proc. Natl. Acad. Sci. USA* **71**, 1250–1254.
2. Jaenisch, R. (1976) Germ line integration and mendelian transmission of the exogenous Moloney leukemia virus. *Proc. Natl. Acad. Sci. USA* **73**, 1260–1264.
3. Jaenisch, R. (1977) Germ line integration of Moloney leukemia virus. Effect of homozygosity at the M-MuLV locus. *Cell* **12**, 691–696.
4. Gordon, J. W. and Ruddle, F. H. (1983) Gene transfer into mouse embryos: production of transgenic mice by pronuclear injection. *Methods Enzymol.* **101**, 411–433.
5. Brinster, R. L. (1993) Stem cells and transgenic mice in the study of development. *Int. J. Dev. Biol.* **37**, 89–99.
6. Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A., and Ruddle, F. H. (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. USA* **77**, 7380–7384.
7. Brinster, R. L., Chen, Y., Trumbauer, M., Senear, A. W., Warren, R., and Palmiter, R. D. (1981) Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* **27**, 223–231.
8. Constantini, F. and Lacy, E. (1981) Introduction of a rabbit B-globin gene into the mouse germ line. *Nature* **294**, 92–94.
9. Harbers, K., Jahner, D., and Jaenisch, R. (1981) Microinjection of cloned retroviral genomes into mouse zygotes: integration and expression in the animal. *Nature* **293**, 540–542.
10. Wagner, E. F., Stewart, T. A., and Mintz, B. (1981) The human B-globin gene and a functional viral thymidine kinase gene in developing mice. *Proc. Natl. Acad. Sci. USA* **78**, 5016–5020.
11. Wagner, T. E., Hoppe, P. C., Jollick, J. D., Scholl, D. R., Hodinka, R. L., and Gault, J. B. (1981) Microinjection of a rabbit B-globin gene into zygotes and its

- subsequent expression in adult mice and their offspring. *Proc. Natl. Acad. Sci. USA* **78**, 6376–6380. 2
12. Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. J., Birnberg, N. C., and Evans, R. M. (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* **300**, 611–615. 3
 13. Palmiter, R. D. and Brinster, R. L. (1986) Germ-line transformation of mice. *Annu. Rev. Genet.* **20**, 465–499. 3
 14. Jaenisch, R. (1988) Transgenic animals. *Science* **240**, 1468–1474.
 15. Hanahan, D. (1989) Transgenic mice as probes into complex systems. *Science* **246**, 1265–1275. 3
 16. Wagner, E. F. and Keller, G. (1992) The introduction of genes into mouse embryos and stem cells, in *Development: The Molecular Genetic Approach* (Russo, V. E. A., Brody, S., Cove, D., and Ottolenghi, S., eds.), Springer-Verlag, Berlin and Heidelberg, pp. 440–458. 3
 17. Wagner, E. F. (1990) On transferring genes into stem cells and mice. *EMBO J.* **9**, 3025–3032. 3
 18. Cameron, E. R., Harvey, M. J. A., and Onions, D. E. (1994) Transgenic science. *Br. Vet. J.* **150**, 9–24.
 19. Mansour, S. L., Thomas, K. R., and Capecchi, M. R. (1988) Disruption of the protooncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336**, 348–352. 3
 20. Capecchi, M. R. (1989) Altering the genome by homologous recombination. *Science* **244**, 1288–1292. 3
 21. Zijlstra, M. R., Li, E., Sajjadi, F., Subramani, S., and Jaenisch, R. (1989) Germ-line transmission of a disrupted B2 microglobulin gene produced by homologous recombination in embryonic stem cells. *Nature* **342**, 435–438. 3
 22. DeChiara, T. M., Efstratiadis, A., and Robertson, E. J. (1990) A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* **345**, 78–80. 3
 23. Koller, B. H., Marruck, P., Kappler, J. W., and Smithies, O. (1990) Normal development of mice deficient in B2M MHC class I proteins, and CD8+ T cells. *Science* **248**, 1227–1230. 3
 24. Fassler, R., Martin, K., Forsberg, E., Litzzenburger, T., and Iglesias, A. (1995) Knockout mice: how to make them and why. The immunological approach. *Int. Arch. Allergy Immunol.* **106**, 323–334. 4
 25. Piérce, G. B., Jr. (1975) Teratocarcinoma: introduction and perspectives, in *Teratomas and Differentiation* (Sherman, M. I. and Softer, D., eds.), Academic, New York, pp. 3–12. 4
 26. Dewey, M. J., Martin, D. W., Jr., Martin, G. R., and Mintz, B. (1977) Mosaic mice with teratocarcinoma-derived mutant cells deficient in hypoxanthin phosphoribosyl transferase. *Proc. Natl. Acad. Sci. USA* **74**, 5564–5568. 4
 27. Thomas, K. R. and Capecchi, M. R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503–512. 4

28. Brinster, R. L. and Zimmerman, J. W. (1994) Spermatogenesis following male germ cell transplantation. *Proc. Natl. Acad. Sci. USA* **91**, 11,298–11,302.
29. Brinster, R. L. and Avarbock, M. R. (1994) Germline transmission of donor haplotype following spermatogonial transplantation. *Proc. Natl. Acad. Sci. USA* **91**, 11,303–11,307.
30. Dym, M. (1994) Spermatogonial stem cells of the testes. *Proc. Natl. Acad. Sci. USA* **91**, 11,287–11,289.
31. Brinster, R. L., Chen, H. Y., Trumbauer, M. E., Yagle, M. K., and Palmiter, R. D. (1985) Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc. Natl. Acad. Sci. USA* **52**, 4438–4442.
32. Grigoriadis, A. E., Wang, Z. Q., and Wagner, E. F. (1993) c-fos oncogene expression in cartilage and bone tissues of transgenic and chimeric mice, in *Cellular and Molecular Biology of Bone* (Noda, M., ed.), Academic, London, pp. 497–537.
33. Palmiter, R. D., Chen, H. Y., and Brinster, R. L. (1982) Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. *Cell* **29**, 701–710.
34. Kuehn, M. R. and Stoye, J. P. (1992) Insertional mutagenesis and mouse development, in *Development: The Molecular Genetic Approach* (Russo, V. E. A., Brody, S., Cove, D., and Ottolenghi, S., eds.), Springer-Verlag, Berlin and Heidelberg, pp. 420–439.
35. Soriano, P. and Jaenisch, R. (1986) Retroviruses as probes for mammalian development: allocation of cells to the somatic and germ cell lineages. *Cell* **46**, 19–29.
36. Brinster, R. L., Chen, H. Y., Warren, R., Sarthy, A., and Palmiter, R. D. (1982) Regulation of metallothionein-thymidine kinase fusion plasmids injected into mouse eggs. *Nature* **296**, 39–42.
37. Brinster, R. L. and Palmiter, R. D. (1986) Introduction of genes into the germ line of animals. *Harvey Lect.* **80**, 1–38.
38. Niederreither, K., D'Souza, R. N., and De Crombrughe, B. (1992) Minimal DNA sequences that control the cell lineage-specific expression of the pro α 2(1) collagen promoter in transgenic mice. *J. Cell Biol.* **119**, 1361–1370.
39. D'Souza, R. N., Niederreither, K., and De Crombrughe, B. (1993) Osteoblast-specific expression of the α 2(I) collagen promoter in transgenic mice: correlation with the distribution of TGF- β 1. *J. Bone Min. Res.* **8**, 1127–1136.
40. Cui, C., Wani, M. A., Wight, D., Kopchick, J., and Stambrook, P. J. (1994) Reporter genes in transgenic mice. *Transgenic Res.* **3**, 182–194.
41. Bonnerot, C. and Nicolas, J.-F. (1993) Application of lac Z gene fusions to postimplantation development. *Methods Enzymol.* **225**, 451–472.
42. Jacenko, O., Olsen, B. R., and LuValle, P. (1991) Organization and regulation of collagen genes. *Eukaryotic Gene Expr.* **1**, 327–353.
43. Herskowitz, I. (1987) Functional inactivation of genes by dominant negative mutations. *Nature* **329**, 219–222.
44. Westphal, H. and Gruss, P. (1989) Molecular genetics of development studied in the transgenic mouse. *Ann. Rev. Cell Biol.* **5**, 181–196.

45. Izant, J. G. and Weintraub, H. (1984) Inhibition of thymidine kinase gene expression by anti-sense RNA: a molecular approach to gene analysis. *Cell* 36, 1007-1015.
46. Izant, J. G. and Weintraub, H. (1985) Constitutive and conditional suppression of exogenous and endogenous genes by anti-sense RNA. *Science* 229, 345-352.
47. Rosenberg, U. B., Preiss, A., Seifert, H. E., Jackle, H., and Knipple, D. C. (1985) Production of phenocopies by kruppel anti-sense RNA injection into drosophila embryos. *Nature* 313, 703-706.
48. Melton, D. A. (1985) Injected anti-sense RNAs specifically block messenger RNA translation in vivo. *Proc. Natl. Acad. Sci. USA* 82, 144-148.
49. Katsuki, M., Sato, M., Kimura, M., Yokoyama, M., Kobayashi, K., and Nomura, T. (1988) Conversion of myelin basic protein antisense cDNA in transgenic mice. *Science* 241, 593-595.
50. Simons, M., Edelman, E. R., Dekeyser, J. L., Langer, R., and Rosenberg, R. D. (1992) Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. *Nature* 359, 67-70.
51. Jacenko, O., Olsen, B. R., and Warman, M. L. (1994) Of mice and men: heritable skeletal disorders. *Am. J. Hum. Genet.* 54, 163-168.
52. Jacenko, O. and Olsen, B. R. (1995) Transgenic mouse models in studies of skeletal disorders. *J. Rheumatol.* 22, 39-41.
53. Stacy, A., Bateman, J., Choi, T., Mascara, T., Cole, W., and Jaenisch, R. (1988) Perinatal lethal osteogenesis imperfecta in transgenic mice bearing an engineered mutant pro- $\alpha 1$ (I) collagen gene. *Nature* 332, 131-135.
54. Prockop, D. J., Baldwin, C. T., and Constantinou, C. D. (1990) Mutations in type I procollagen genes that cause osteogenesis imperfecta. *Adv. Hum. Genet.* 19, 105-132.
55. Hammer, R. E., Brinster, R. L., and Rosenfield, M. G. (1985) Expression of human growth hormone-releasing factor in transgenic mice results in increased somatic growth. *Nature* 315, 413-416.
56. Palmiter, R. D., Behringer, R. R., Quaife, C. J., Maxwell, F., Maxwell, I. H., and Brinster, R. L. (1987) Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. *Cell* 50, 435-443.
57. Behringer, R. R., Mathews, R. D., Palmiter, R. D., and Brinster, R. L. (1988) Dwarf mice produced by genetic ablation of growth hormone-expressing cells. *Genes Dev.* 2, 453-544.
58. Bruggeman, L. A., Xie, H. X., Brown, K. S., and Yamada, Y. Y. (1991) Developmental regulation for collagen II gene expression in transgenic mice. *Teratol.* 44, 203-208.
59. Camper, S. A., Saunders, T. L., Kendall, S. K., Keri, R. A., Seasholtz, A. F., Gordon, D. F., Birkmeier, T. S., Keegan, C. E., Karolyi, I. J., Roller, M. L., Burrows, H. L., Samuelson, L. C. (1995) Implementing transgenic and embryonic stem cell technology to study gene expression, cell-cell interactions and gene function. *Biol. Reprod.* 52, 246-257.
60. Borrelli, E., Heyman, R. A., Arias, C., Sawchenko, P. E., and Evans, R. M. (1989) Transgenic mice with inducible dwarfism. *Nature* 339, 538-544.

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61. Vaux, D. L. (1993) Toward an understanding of the molecular mechanisms of physiological cell death. *Proc. Natl. Acad. Sci. USA* **90**, 786–789.
62. Weissman, I. L. (1994) Developmental switches in the immune system. *Cell* **76**, 207–218.
63. Rossant, J. and Hopkins, N. (1992) Of fin and fur: mutational analysis of vertebrate embryonic development. *Genes Dev.* **6**, 1–13.
64. Woychik, R. B., Stewart, T. A., Davis, L. G., D'Eustachio, P., and Leder, P. (1985) An inherited limb deformity created by insertional mutagenesis in a transgenic mouse. *Nature* **318**, 36–40.
65. Schnieke, A., Harbers, K., and Jaenisch, R. (1984) Insertion of retrovirus into the first intron of $\alpha 1(I)$ collagen gene. *Nature* **304**, 315–320.
66. Hartung, S., Jaenisch, R., and Breindl, M. (1986) Retrovirus insertion inactivates mouse $\alpha 1(I)$ collagen gene by blocking initiation of transcription. *Nature* **320**, 365,366.
67. Gossler, A., Joyner, A. L., Rossant, J., and Skarnes, W. C. (1989) Mouse embryonic stem cells and reporter constructs to detect developmentally regulated genes. *Science* **244**, 463–465.
68. Rijkers, T., Peetz, A., and Ruther, U. (1994) Insertional mutagenesis in transgenic mice. *Transgenic Res.* **3**, 203–215.
69. Gossen, J. A., de Leeuw, W. J., and Vijg, J. (1994) LacZ transgenic mouse models: their application in genetic toxicology. *Mutation Res.* **307**, 451–459.
70. Jacenko, O., LuValle, P., and Olsen, B. R. (1993) Spondylometaphyseal dysplasia in mice carrying a dominant negative mutation in a matrix protein specific for cartilage-to-bone transition. *Nature* **365**, 56–61.
71. Keller, G., Paige, C., Gilboa, E., and Wagner, E. F. (1985) Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. *Nature* **318**, 149–154.
72. van der Putten, H., Botteri, F. M., Miller, A. D., Rosenfeld, M. G., Fan, H., Evans, R. M., and Verma, I. M. (1985) Efficient insertion of genes into the mouse germline via retroviral vectors. *Proc. Natl. Acad. Sci. USA* **82**, 6148–6152.
73. Stewart, C. L., Ruther, U., Garber, C., Vanek, M., and Wagner, E. F. (1986) The expression of retroviral vectors in murine stem cells and transgenic mice. *J. Embryol. Exp. Morphol.* **97 Suppl.**, 263–275.
74. Temin, H. M. (1989) Retrovirus vectors: promise and reality. *Science* **246**, 983.
75. Capecchi, M. (1990) Tapping the cellular phone. *Nature* **344**, 105.
76. Robertson, E. J. (1987) Embryo-derived stem cell lines, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (Robertson, E. J., ed.), IRL, Oxford, pp. 71–112.
77. Valancius, V. and Smithies, O. (1991) Testing an “in-out” target procedure for making subtle genomic modifications in mouse embryonic stem cells. *Mol. Cell. Biol.* **11**, 1402–1408.
78. Hasty, P., Ramirez-Solis, R., Krumlauf, R., and Bradley, A. (1991) Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature* **350**, 243–246.

79. Wood, S. A., Allen, N. D., Rossant, J., Auerbach, A., and Nagy, A. (1993) Non-injection methods for the production of embryonic stem cell-embryo chimeras. *Nature* 365, 87-89.
80. Thomas, K. R. and Capecchi, M. R. (1990) Targeted disruption of the murine *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* 346, 847-850.
81. McMahon, A. P. and Bradley, A. (1990) The Wnt-1 (*int-1*) proto-oncogene is required for development of a large region of the mouse brain. *Cell* 62, 1073-1085.
82. Wang, Z. Q., Ovitt, C., Grigoriadis, A. E., Steinlein, U. M., Ruther, U., and Wagner, E. F. (1992) Bone and hematopoietic defects in mice lacking *c-fos*. *Nature* 360, 742-745.
83. Johnson, R. S., Spiegelman, B. M., and Papaloannou, V. (1992) Pleiotropic effects of a null mutation in the *c-fos* proto-oncogene. *Cell* 71, 577-586.
84. Grigoriadis, A. E., Wang, Z. Q., Cecchini, M. G., Hofstetter, W., Felix, R., Fleisch, H. A., and Wagner, E. F. (1994) *c-Fos*: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* 266, 443-448.
85. Jacenko, O. (1995) *c-fos* and bone loss: a regulator of osteoclast lineage determination. *BioEssays* 17, 277-281.
86. Rhim, J. A., Sandgren, E. P., Degen, J. L., Palmiter, D. R. D., and Brinster, R. L. (1994) Replacement of diseased mouse liver by hepatic cell transplantation. *Science* 263, 1149-1151.
87. Heckel, J. L., Sandgren, E. P., Degen, J. L., Palmiter, R. D., and Brinster, R. L. (1990) Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell* 62, 447-456.

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